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13. ABSTRACT ***(Maximum 200 Words)***

Women who inherit a BRCA1 or BRCA2 mutation have an 80-90% chance of developing breast cancer. Gene targeting techniques were used to create a Brca2-deficient mouse. A portion of exon 10 was replaced with the Neo gene resulting in premature truncation of the protein product. Examination of normal mammary ductal development in $129^{(+/+)}$ and $129^{(+/Brca2-)}$ mice revealed phenotypic differences between the two genotypic classes. $129^{(+/+)}$ and $129^{(+/Brca2-)}$ mice and several inbred mouse strains were used to study the effect of genetic background on radiation induced mammary tumor induction. BALB/c and SWR mice are susceptible to radiation induced mammary tumorigenesis and C57BL/6, FVB/N and C3H mice are relatively resistant. While FVB/N mice do not develop tumors, radiation treatment has a dramatic effect on ductal morphogenesis. To date, no mammary tumors have been observed in the $129^{(+/+)}$ and $129^{(+/Brca2-)}$ mice. Brca2-deficient mice were crossed to p53 mutant mice to generate four genotypic classes of offspring. A study is in progress to assess the consequences of harboring mutation in the two tumor suppressor genes with and without radiation exposure. The mammary tumor susceptible BALB/c and resistant C57BL/6 mouse strains being used to make mice congenic for the Brca2 mutation.

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FOREWORD

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Michelle Bennett 8/2/01

PI - Signature Date

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Introduction

Approximately 180,000 women will be diagnosed with breast cancer this year and 43,000 women will die from the disease. It is the most commonly diagnosed cancer and the second leading cause of cancer deaths among women. Approximately 7% of breast cancers are attributed to the inheritance of BRCA mutations. Women who inherit a mutated copy of the BRCA2 gene have a 28% chance of developing breast cancer before the age of 50 and a lifetime risk that has been determined to be as high as 85%. In order to better understand the consequences of inheriting an alteration in the BRCA2 gene I proposed to develop a mouse model using gene targeting technology. $129^{(+/Brca2-)}$ deficient mice were created with a targeted mutation in exon 10 of the endogenous mouse Brca2 gene. The study objectives for the use of these mice were to: (a) assess differences in normal and neoplastic growth control in mice carrying one or two defective copies of the BRCA2 gene, (b) determine the cancer risks of radiation exposure in mice with BRCA2 defects, and (c) study the effect of carrying defects in two tumor suppressor genes by mating BRCA2-deficient mice with transgenic mice that carry a mutant copy (Ala135Val) of the p53 tumor suppressor gene. These studies were designed to understand potential gene-environment and gene-gene interactions. It was of particular interest to study germline Brca2 mutations in association with radiation, a known breast carcinogen, and a mutated p53 gene, which when inherited also predisposes women to breast cancer development.

Body

Specific Aim 1:

Development of a Brca-2 deficient mouse

As described in the 1999 Annual Report, this aim of the proposal has been completed. As described in the 2000 Annual Report these mice have been used to address the objectives proposed in the originally submitted grant application, as well as other studies.

Specific Aim 2:

Information Described in the 2000 Annual Report

1. It was determined that Brca2-null mice on the BALB/c genetic background die at embryonic day 10.5, two days later than on the 129/SvEv background
2. The two-year study to evaluate the response of several inbred mouse strains to mammary tumor induction by low-level radiation has been completed. The tissues from C57BL/6NCI, BALB/cJ, C3H/HeNCI, SWR/J, and FVB/N inbred mouse strains are being evaluated.
3. The two year final sacrifices began in the fall of 1999 and there were less than 10 mice left to be sacrificed. Gross pathology had not been observed that distinguished mice with and without a Brca2 mutation. However conclusions will not be made until pathology and diagnosis have been completed.

Current and Extended Results

Animals

All the animals that were used in these experiments have been euthanized. There are no living animals left to be euthanized for this study. The animals are represented by tissues and mammary gland whole mounts preserved for microscopic and macroscopic analysis.

Radiation induced mammary tumorigenesis in inbred mouse strains

The data for this portion of the aim have been collected. An application for the transfer of the remaining funds for this proposal has been submitted to the DOD in addition for a request for a one-year no-cost extension. If the application for the transfer of funds to LLNL and a no-cost extension is approved by the DOD, a manuscript will be prepared to describe the effects of whole body radiation in the C3H/HeNCI, C57BL/6NCI, FVB/N, BALB/cJ and SWR/J inbred mouse strains.

Does radiation exposure of $129^{(+/Brca2-)}$ mice increase their risk for mammary tumor induction?

Some slides have been prepared and are waiting pathological evaluation and diagnosis by John Seely and Barb Davis (NIEHS). From there we will be able to perform statistical analyses and determine if there are any differences among the treated and control groups with and without a Brca2 mutation. At the current time there is no evidence for increased risk to the $129^{(+/Brca2-)}$ mice.

Specific Aim 3:

Reported in the 2000 Annual Report

1. To date, we have not observed an increased incidence of mammary tumors in the FVB129F1$^{(+/Brca2-)}$, FVB129F1$^{(p53mut/Brca2-)}$, or FVB129F1$^{(p53mut/+)}$ compared to one another or the wild type FVB129F1$^{(+/+)}$ littermates. In addition, no other gross pathology, apparent at necropsy, distinguishes the genotypic classes. Partial or full necropsies were performed on the mice that became moribund during the course of the experiment. All the tissues from the mice are being processed for routine histology

Current Status

All tissues from the mice have been collected, and are awaiting analysis and diagnosis. Once the data has been compiled it will be evaluated statistically. The fourth abdominal mammary glands have been stained and mounted to slides for morphologic assessment.

Specific Aim 4:

Information Described in the 2000 Annual Report

The Brca2 mutation was made congenic on the BALB/c genetic background. BALB/c mice are susceptible to radiation-induced mammary tumorigenesis and radiation-induced changes in mammary ductal morphology after relatively low exposures.

Summary:

The work proposed in this grant application is near completion. We have data on the many animals that were included in Specific Aims 2 and 3. However, because there has been no funding for this proposed work since early September 2000 (see below), final or re-analyses of tissue has not been completed. As a result data have not been fully collected or analyzed. The preparation of manuscripts for these studies are pending the completion of these tasks.

Key Research Accomplishments

The funding for this grant was stopped in September 2000. Dr. Michelle Bennett, in keeping with the philosophy of the DOD's postdoctoral training grant mechanism, secured an independent position in which she is currently trying to establish herself as an independent investigator in the field of Breast Cancer Research. Upon her move to Lawrence Livermore National Laboratory (LLNL) to begin her new position she was informed by the USAMRMC she could apply to have the funds remaining from her postdoctoral training grant transferred to LLNL. Dr. Bennett completed and submitted the required paperwork in December of 2000.

At this time Dr. Bennett was advised to submit a request for a no-cost extension because the process of review for the transfer of funds might take several months. Dr. Bennett submitted a request for a no-cost extension for this study in December 2000.

In April 2001 Dr. Bennett inquired about the status of the potential transfer of funds and was told that all the required documentation was in the hands of the DOD except for a copy of the current Institutional Animal Care and Use Committee approved Animal Protocol for the funded study. Dr. Bennett explained that she had submitted the Approved Animal Protocol that covered the use of all animals in the study which originated at NIEHS, NIH where she did her postdoctoral training. All Animals in the study were housed and sacrificed at NIEHS. All animals were sacrificed before Dr. Bennett's move to LLNL. As a result, there were no animals for which to prepare an animal protocol for the Institutional Animal Care and Use Committee at LLNL. Dr. Bennett did not seek approval for the use of the animals that were used in this study because there were no live animals left in the study.

Very little has been done for this study in the last year. The final analyses, data collection and manuscript preparation need to be done. This work is currently pending the outcome of this situation. Recent interactions with people at the USAMRAA have been positive and I am optimistic that the situation will be resolved soon.

List of Reportable Outcomes

Papers

Paper described as "in press" in the 2000 Annual Report were published:

Brca2-Null Embryonic Survival is Prolonged on the BALB/c Genetic Background. L. Michelle Bennett, Kimberly A. McAllister, Pamela E. Blackshear, Jason Malphurs, Gina Goulding, N. Keith Collins, Toni Ward, Donna O. Bunch, Edward M. Eddy, Barbara J. Davis, and Roger W. Wiseman. Molecular Carcinogenesis, 28:174-183, 2000.

Mice heterozygous for a *Brca1* or *Brca2* mutation display distinct mammary gland and ovarian phenotypes in response to diethylstilbestrol. L. Michelle Bennett, Kimberly A. McAllister, Jason Malphurs, Toni Ward, N. Keith Collins, John C. Seely, Lori C. Gowen, Beverly H. Koller, Barbara J. Davis, and Roger W. Wiseman. Cancer Research, 60: 3461-3469, 2000.

In addition a paper that used the C57BL/6 strain congenic for the Brca2 mutation was published: Mammary Tumor Induction and Premature Ovarian Failure in Apc^{Min} Mice are Not Enhanced by *Brca2* Deficiency. Bennett, L.M., McAllister, K.A., Ward, T., Malphurs, J., Collins, N.K., Seely, J.C., Davis, B.J., and Wiseman, R.W.. *Toxicologic Pathology*, 29: 117-125, 2001.

Funding Applications

Dr. Bennett has prepared three grant applications as P.I. and helped prepare another as a collaborator in which data obtained from this funded study were included in the preliminary data section. These include:

1. Grant application to the California Breast Cancer Research Program January 11, 2001. Dr. Bennett has been notified by the agency that this grant application was selected for funding. Funds have not yet been received.
2. Grant Application by Dr. James Tucker (LLNL) in response to the DOE National Laboratories, LAB 01-18, Low Dose Radiation Research Program, May 15, 2001.
3. IDEA Grant application to the Department of Defense Breast Cancer Research Program - Program Announcement II, June 13, 2001.
4. Career Development Award Grant application to the Department of Defense Breast Cancer Research Program - Program Announcement II, June 13, 2001.

Accepted Job Offer

Dr. Bennett began her new position at Lawrence Livermore National Laboratory, Livermore, California September 5, 2000.

Conclusions

A mouse that is heterozygous for a Brca2 mutation has been developed on the BALB/c, 129 and C57BL/6 genetic backgrounds. Strain 129 mice that inherit a mutated copy of Brca2 do not appear to be predisposed to an increased incidence of spontaneous or radiation-induced mammary tumors compared to wild type controls. When two copies of the Brca2 mutation are inherited on the129 or a mixed 129 and C57BL/6 background the embryos die at d8.5 gestation. BALB/c mice that have inherited two mutant copies of Brca2 are also embryonic lethal. However, we have determined that the day of death is prolonged to d10.5 on the BALB/c background. Numerous samples are waiting final analysis in anticipation of preparing manuscripts to described this funded research.

References

Brca2-Null Embryonic Survival is Prolonged on the BALB/c Genetic Background. L. Michelle Bennett, Kimberly A. McAllister, Pamela E. Blackshear, Jason Malphurs, Gina Goulding, N. Keith Collins, Toni Ward, Donna O. Bunch, Edward M. Eddy, Barbara J. Davis, and Roger W. Wiseman. *Molecular Carcinogenesis*, 28:174-183, 2000.

Mice heterozygous for a *Brca1* or *Brca2* mutation display distinct mammary gland and ovarian phenotypes in response to diethylstilbestrol. L. Michelle Bennett, Kimberly A. McAllister, Jason Malphurs, Toni Ward, N. Keith Collins, John C. Seely, Lori C. Gowen, Beverly H. Koller, Barbara J. Davis, and Roger W. Wiseman. *Cancer Research*, 60: 3461-3469, 2000.

Mammary Tumor Induction and Premature Ovarian Failure in Apc^{Min} Mice are Not Enhanced by *Brca2* Deficiency. L. Michelle Bennett, Kimberly A. McAllister, Toni Ward, Jason Malphurs, Keith Collins, John C. Seely, Barbara J. Davis, and Roger W. Wiseman. *Toxicologic Pathology*, 29: 117-125, 2001.

Appendix

Mammary Tumor Induction and Premature Ovarian Failure in Apc^{Min} Mice are Not Enhanced by *Brca2* Deficiency. L. Michelle Bennett, Kimberly A. McAllister, Toni Ward, Jason Malphurs, Keith Collins, John C. Seely, Barbara J. Davis, and Roger W. Wiseman. *Toxicologic Pathology*, 29: 117-125, 2001.

TOXICOLOGIC PATHOLOGY, vol 29, no 1, pp 117–125, 2001

Mammary Tumor Induction and Premature Ovarian Failure in *Apc*^{Min} Mice Are Not Enhanced by *Brca2* Deficiency

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ABSTRACT

Inherited *BRCA2* mutations predispose individuals to breast cancer and increase risk at other sites. Recent studies have suggested a role for the *APC* I1307K allele as a low-penetrance breast cancer susceptibility gene that enhances the phenotypic effects of *BRCA1* and *BRCA2* mutations. To model the consequences of inheriting mutant alleles of the *BRCA2* and *APC* tumor suppressor genes, we examined tumor outcome in C57BL/6 mice with mutations in the *Brca2* and *Apc* genes. We hypothesized that if the *Brca2* and *Apc* genes were interacting to influence mammary tumor susceptibility, then mammary tumor incidence and/or multiplicity would be altered in mice that had inherited mutations in both genes. Female and male offspring treated with a single IP injection of 50 mg/kg *N*-ethyl-*N*-nitrosourea (ENU) at 35 days of age developed mammary adenoacanthomas by 100 days of age. The female *Apc*-mutant and *Brca2*/*Apc* double-mutant progeny had mean mammary tumor multiplicities of 6.7 ± 2.8 and 7.2 ± 2.7, respectively, compared to wild-type and *Brca2*-mutant females, which had mean mammary tumor multiplicities of 0.1 ± 0.4 and 0.3 ± 0.5, respectively. Female ENU-treated *Apc*-mutant and *Brca2*/*Apc* double heterozygotes were also susceptible to premature ovarian failure. Thus, the inheritance of an *Apc* mutation predisposes ENU-treated female and male mice to mammary tumors and, in the case of female mice, to ovarian failure. These results indicate that mammary tumor development in *Apc*-mutant mice can progress independently of ovarian hormones. The *Apc* mutation-driven phenotypes were not modified by mutation of *Brca2*, perhaps because *Brca2* acts in a hormonally dependent pathway of mammary carcinogenesis.

Keywords. Mammary ducts; morphology; breast cancer; adenoacanthoma; hormone; *Wnt* pathway

INTRODUCTION

Inherited mutations in the human breast cancer susceptibility gene, *BRCA2*, profoundly predispose women to the development of breast cancer. Women who inherit mutations in the *BRCA2* gene have been reported to have a lifetime risk for breast cancer as high as 85% (32), while for men the risk is approximately 7% (9). In addition, *BRCA2* alterations have been associated with cancer susceptibility at other sites, including the ovary, stomach, larynx, colon, and prostate (41). While the function of the *BRCA2* protein has yet to be elucidated, it is believed to play a role in tumor suppression, in DNA damage repair, and in the maintenance of genomic stability (22, 37, 40).

Genes in the *Wnt/APC/β-catenin*–signaling pathway have been evaluated for their association with breast cancer development (1, 4, 10, 19, 41). The *APC* gene has been suggested to be a low-penetrance breast cancer susceptibility gene or a modifier of the *BRCA* loci (33, 45) in addition to its well-established role in familial and sporadic colon cancer (14, 29). Breast cancer patients who inherit the I1307K polymorphic *APC* allele are twice as likely to carry a mutation in one of the breast cancer susceptibility genes, *BRCA1* or *BRCA2*, compared to the general population. The *APC* I1307K allele polymorphism occurs in approximately 7% of the Ashkenazi Jewish population. Caused by an inherited T-to-A transversion at nucleotide 3920, this polymorphism creates an unstable poly A tract that is susceptible to mutation, apparently by polymerase slippage during DNA replication (21). Such polymerase slippage is hypothesized to cause frameshift mutations that disrupt the APC gene product.

Brca2 and *Apc* mouse models have been developed to study the consequences of inherited mutations in these tumor suppressor genes. There have been no reports of increased mammary tumor development, either spontaneous or induced, in mouse models created to study mutations in the endogenous *Brca2* gene (15). However, two *Apc*-mutant mouse strains are susceptible to carcinogen-induced mammary and ovarian tumor development (27, 43). *Apc*-mutant *Min*/+ mice carry a nonsense mutation at codon 850 on a C57BL/6 genetic background (C57BL/6$^{(Apc\pm)}$ and are susceptible to mammary tumor induction by the alkylating agent *N*-ethyl-*N*-nitrosourea (ENU) (26, 27). Female *Apc*1638N mutant mice on a C56BL/6 genetic background display a 16-fold increase in mammary tumor incidence after treatment with 5 Gy radiation at 7 weeks of age (43). In addition, radiation-treated (BALB/c × *Apc*1638N)F1 mice developed ovarian tumors, while F1s from crosses with several other inbred strains did not (44). Thus, genetic modifiers from different inbred strain backgrounds influence susceptibility to ovarian cancer in *Apc*1638N mice (44).

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TABLE 1.—Mean initial and final body weights for the four genotypic classes.

Genotype	Initial body weight[a]		Final body weight[a]		Δ Body weight[a]	
	N	Mean (SD)	*N*	Mean (SD)	*N*	Mean (SD)
Brca2$^{+/+}$*Apc*$^{+/+}$	33	16.7 (1.3)	16[b]	22.5 (3.0)	16	5.3 (2.4)
Brca2$^{+/-}$*Apc*$^{+/+}$	35	16.8 (1.0)	23[b]	22.4 (1.7)	23	5.5 (1.2)
Brca2$^{+/+}$*Apc*$^{+/-}$	36	16.3 (1.2)	26[b]	20.4 (3.1)[c]	26	3.8 (3.1)[c]
Brca2$^{+/-}$*Apc*$^{+/-}$	27	16.4 (1.1)	23[b]	21.4 (1.9)[d]	23	4.9 (2.1)[d]

[a] Body weight is reported in grams.
[b] Final body weights were not available for all animals.
[c] Mean value is significantly different than corresponding wild-type and *Brca2*-deficient classes ($p < 0.05$ by Fisher's LSD test).
[d] Mean value is not statistically different from corresponding wild-type, *Brca2*-deficient or *Apc*-mutant classes.

ranged from 16.3 ± 1.2 to 16.8 ± 1.0 and were not statistically different (Table 1). However, at the end of the experiment, there was some variation in the mean body weight values among the 4 genotypic classes. The average weight at sacrifice was calculated for each of the genotypic classes: wild type, 22.5 ± 3.0; *Brca2* deficient, 22.4 ± 1.7; *Apc* mutant, 20.4 ± 3.1; and double heterozygote, 21.4 ± 1.9. Analysis of variance (ANOVA) indicated a significant effect from the *Apc* mutation. Body weight was not affected by the inheritance of a *Brca2* mutation, and no interaction was observed between *Apc* and *Brca2* with regard to body weight or weight gain. Pairwise comparisons using Fisher's LSD test indicated a statistical difference between the *Apc*-mutant animals and the wild-type and *Brca2*-deficient animals ($p < 0.05$). However, the *Brca2/Apc* double-heterozygous class did not differ significantly from the other groups. Thus, the presence of an *Apc* mutation did not have a predominant influence on body weight in this experiment.

Mammary Tumor Incidence and Multiplicity in C57BL/6$^{(B2\pm)}$ × *C57BL/6*$^{(Apc\pm)}$ *Male and Female Offspring*

Female mice that inherited an *Apc* mutation developed mammary tumors with 100% incidence with an average multiplicity of 6.7 ± 2.8 65 days after ENU treatment (Table 2 and Figure 1). ENU-treated female mice with inherited mutations in both *Brca2* and *Apc* genes also developed mammary tumors with 100% incidence and a mammary tumor multiplicity of 7.2 ± 2.7, which was not statistically different from mice with an *Apc* mutation alone. There were no observed differences in tumor size distribution between the *Apc*- and *Brca2/Apc* double mutants. The wild-type and *Brca2*-deficient mice were relatively resistant to mammary tumor induction by ENU. Female wild-type and *Brca2*-deficient mice had mammary tumor incidences of 7% and 23%, respectively. The female wild-type mice had a mean mammary tumor multiplicity of 0.1 ± 0.4, and the *Brca2*-deficient group had a mean mammary tumor multiplicity of 0.3 ± 0.5. The tumor multiplicities observed in the females harboring an *Apc* mutation was statistically different than those observed in the wild-type and *Brca2*-deficient genotypic classes ($p < 0.001$).

Mutations in the human *BRCA2* gene have been associated with male breast cancer (9). Male mice representing all 4 genotypic classes were included in the experiment to assess the effect of the *Brca2* and *Apc* mutations on mammary tumor susceptibility. Male mice that inherited the *Apc* mutations, alone or in combination with a *Brca2* alteration, developed mammary tumors with a very low incidence (Table 2). Male *Apc*-mutant and *Brca2/Apc* double-mutant mice had mean tumor multiplicities of 0.4 ± 0.5 and 0.2 ± 0.3, respectively. The male wild-type and *Brca2*-deficient mice did not develop any mammary tumors. The difference in tumor multiplicities among male mice of the different genotypic classes was not significant. However, the development of mammary tumors with a short latency in male mice with an inherited *Apc Min* mutation may be biologically relevant.

Histologic Evaluation of Mammary Tumors

The mammary tumors examined from both male and female mice were characterized as adenoacanthomas and are the same histologic type as observed in previous studies of mammary tumor development in *Apc* mutant mice

TABLE 2.—Induction of mammary tumors in male and female C57BL/6$^{B2+/-}$ × C57BL/6$^{Apc+/-}$ F1 animals treated with ENU[a].

Genotype	Number of mice	Mammary tumor		Size distribution	
		Incidence	Multiplicity	<5 mm	>5 mm
Brca2$^{+/+}$*Apc*$^{+/+}$	27 female	0.07	1.1 (0.4)[b]	100%	
	11 male	0	0		
Brca2$^{+/-}$*Apc*$^{+/+}$	30 female	0.23	0.3 (0.5)	100%	
	10 male	0	0		
Brca2$^{+/+}$*Apc*$^{+/-}$	36 female	1.00[c]	6.7[d] (2.8)	57%	43%
	11 male	0.36	0.4 (0.5)	50%	50%
Brca2$^{+/-}$*Apc*$^{+/-}$	27 female	1.00[c]	7.2[d] (2.7)	50%	50%
	12 male	0.17	0.2 (0.3)	50%	50%

[a] Animals were treated between 35 and 40 days of age with a single (*IP*) injection of 50 mg/kg ENU. Males and females were sacrificed at 100 days of age.
[b] Values are mean (SD).
[c] Chi-square $p < 0.001$ vs corresponding wild-type and *Brca2*-deficient genotypes.
[d] Two-tailed Mann-Whitney *U* test $p < 0.001$ vs corresponding wild-type and *Brca2*-deficient genotype.

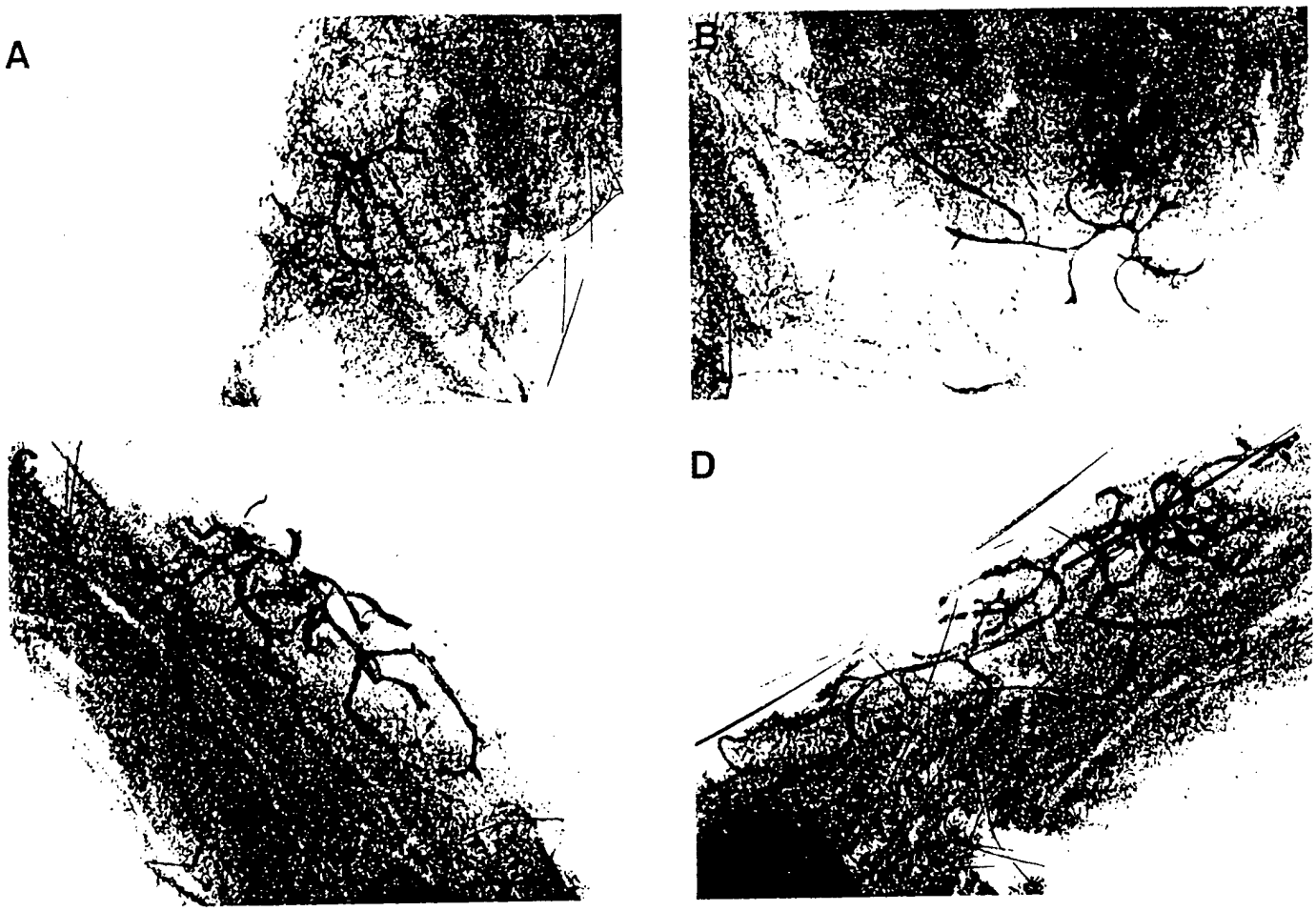

FIGURE 2.—Photomicrograph of mammary gland whole mounts from male mice. Wild-type (A) and *Brca2*-deficient (B) mice display a mammary gland rudiment typical of an adult male. *Apc*-deficient (C) and *Brca2/Apc* double heterozygotes (D) have elongated mammary ducts that extend to the lymph node (arrow) with some relatively complex branching. ×4.2 magnification.

visible within the fat pad (Figure 2A, B). No differences in mammary branching phenotype were noted among the different female groups in this study.

Apc Mutation Carriers Are Predisposed to Premature Ovarian Failure

The overall progression of follicular development in the ovaries isolated from the F1 offspring was assessed.

TABLE 3.—Incidence of reproductive pathologies in ENU-treated female and male C57BL/6$^{Brca2+/-}$ × C57BL/6$^{Apc+/-}$ offspring at 100 days of age.

	Genotype			
Tissue and pathology	*Brca2*$^{+/+}$ *Apc*$^{+/+}$ (N = 26)	*Brca2*$^{+/-}$ *Apc*$^{+/+}$ (N = 27)	*Brca2*$^{+/+}$ *Apc*$^{+/-}$ (N = 35)	*Brca2*$^{+/-}$ *Apc*$^{+/-}$ (N = 27)
Ovary				
Epithelial cyst	0.15	0.04	0.06	0.07
No CLs	0	0.04	0.26[a]	0.22[a]
Atrophy	0	0	0.23[a]	0.22[a]
Uterus				
Atrophy	0	0.04	0.23[a]	0.22[a]

[a] Chi-square $p < 0.05$ vs similarly treated wild-type and *Brca2* +/− genotypic classes

The reproductive tracts of the ENU-treated female mice with *Apc* mutations displayed varying degrees of ovarian histopathology. The Corpora lutea (CL) were absent in 26% of the *Apc*-mutant mice and 22% of the *Brca2/Apc* double mutants (Table 3), and the remaining follicles were typically degenerate. This incidence of arrested follicular development was 6-fold higher than that observed for the *Brca2*-deficient group and was statistically significant ($p < 0.05$). In addition, complete loss of follicles (ovarian atrophy) was observed in approximately one-fourth of the *Apc*-mutant and double-heterozygous mice, while wild-type and *Brca2*-deficient mice rarely displayed this phenotype (Figure 3 and Table 3). The progression from follicular degeneration to complete atrophy was interpreted to represent premature ovarian failure.

In addition, the uteri of the *Apc*-mutant and *Brca2/Apc* double-mutant ENU-treated females with ovarian failure were characterized as atrophic. The uterine endometrium and myometrium appeared quiescent, reminiscent of an immature appearance. It was not clear whether these reproductive organs of the *Apc*-mutant mice had atrophied or had never developed fully. The vaginal epithelium was reduced in thickness and lined by vacuolated cells indicative of anestrus.

been sufficient to induce a maximal mammary tumor response that could be modified only by suppression, such as that which is observed when the C57BL/6 *Apc*-mutant mice are crossed with the resistant AKR/J, CAST/Ei, BALB/cByJ, or C3H/He mouse strains (27, 44). However, it is important to note that, to date, there have been no reports of increased mammary tumor incidence, either spontaneous or carcinogen induced, in female mice heterozygous for a *Brca2* mutation on several genetic backgrounds (15). Thus, it is unclear whether partial or complete loss of *Brca2* will promote carcinogenesis in the mouse mammary gland.

It is also possible that the *Brca2* mutation did not modify *Apc*-driven mammary carcinogenesis because these genes act in different pathways. For example, since tumors developed in the ENU-treated *Apc* mutant male mice and in female mice with or without normal ovarian function, mammary tumor development in *Apc*-mutant mice may progress through a pathway that does not require ovarian hormones. In contrast, because the risk of breast cancer in human *BRCA2* mutation carriers may be increased by the use of oral contraceptives (42) and does not appear to be diminished by parity (18), tumor development likely depends on a hormone-responsive environment. Thus, *Brca2* may not modify *Apc* mutation–dependent mammary carcinogenesis because the *Apc* alteration can influence tumorigenesis without ovarian hormones, while *Brca2* acts in a hormonally dependent pathway.

The first line of evidence supporting the conclusion that ENU-treated *Apc* tumors are not dependent on ovarian hormones is that mammary tumors developed in both ENU-treated female C57BL/$6^{(Apc\pm)}$ and C57BL/$6^{(B2\pm)(Apc\pm)}$ mice, whether ovaries are morphologically normal or are atrophied. Normal ovarian function has been demonstrated to contribute to mammary carcinogenesis in rodents [reviewed in (17)]. However, if mammary tumorigenesis were driven by an endocrine mechanism in this *Apc* model system, then ovarian failure would be expected to be protective. Ovarian failure and degeneration was evident after analysis of the reproductive tracts collected from the female *Apc*-mutant and *Brca2/Apc* double-heterozygous mice. There appeared to be a progression from a cessation of ovulation and CL formation, to failure to ever ovulate and form CLs, to atrophy. This was characterized as premature ovarian failure since *Apc*-mutant and *Brca2/Apc* double-heterozygous mice should be ovulating and forming CLs like their wild-type littermates. Although mammary ductal morphogenesis appeared normal in mice with an *Apc* mutation, the uteri were markedly underdeveloped in mice with ovarian atrophy, providing functional evidence that the ovarian hormones, required for stimulation of uterine development, were lacking.

Because ovarian failure may have been due to changes in body weight, the association between these factors was evaluated. Although the mean body weight for the *Apc*-mutant class was statistically reduced compared to the wild-type and *Brca2*-deficient group, the *Brca2/Apc* double-heterozygous class was not significantly different from the other treatment groups, suggesting that body weight did not play a major role in the observed ovarian phenotype. Instead, it is likely that the *Apc*-mutant mice are genetically predisposed to premature ovarian failure. Other studies have shown that premature ovarian failure may predispose mice to tumor formation (23) and that ovarian tumors did develop in irradiated *Apc*1638N mice on a CB6F1 background (6). In addition, the observed ovarian failure and senile atrophy could have been exacerbated by the ENU treatment of *Apc*-mutant mice. Alkylating agents can cause follicular depletion followed by overproduction of pituitary gonadotropins, which can contribute to aberrant cellular proliferation and tumorigenesis (28). The presence of an *Apc* mutation may be permissive to cell replication in the presence of ENU-induced DNA damage in the ovaries of *Apc*-mutant mice. Further studies will be required to establish the mechanistic basis for the observed ovarian pathology.

Another line of evidence that ovarian hormones may not be required for mammary tumorigenesis in the *Apc* mouse model comes from the observation that male mice developed tumors. While mammary tumor incidence and multiplicity were low in the male mice, tumor development was considered biologically relevant given its rare occurrence in wild-type male mice. In addition, the partial ductal morphogenesis observed in the male mice suggests that mutant *Apc* acts early in mammary gland growth and development. In females and males, normal mammary gland growth is initiated from primordial buds and progresses to the rudimentary structure present at birth. In males, no further development occurs because of the repressive effects of androgens, while in females, mammary ductal morphogenesis and complex branching are induced by the hormonal changes that occur at puberty (17). However, the mammary ducts from ENU-treated male C57BL/$6^{(Apc\pm)}$ and C57BL/$6^{(B2\pm)(Apc\pm)}$ mice are elongated and have a more complex branching structure compared to wild-type and *Brca2*-deficient mice. Thus, the mammary glands of the male ENU-treated *Apc* mutants had partially progressed along a developmental pathway usually reserved for females during puberty and may result from lost repression rather than from hormonal stimulation. Moreover, this partial mammary growth that occurred in males with histologically normal testes likely contributed to tumor sensitivity in the ENU-treated *Apc*-mutant males.

While this study suggests that a *Brca2* alteration does not modify an *Apc* mutation in ENU-treated mice, gene-gene interactions can have striking effects on mammary tumor development in *Apc*-mutant mice. For example, mammary tumor susceptibility in *Apc*-mutant mice is clearly enhanced by the coinheritance of a *Tcf*1 gene mutation, which demonstrates that the *Apc* mutation can accelerate mammary tumorigenesis in the absence of carcinogen treatment. *Tcf*1 is expressed in the basal mammary and proliferating intestinal epithelium and is regulated by *Tcf*4, a component of the *Apc* pathway (34). *Tcf*1 −/− mice are predisposed to spontaneous mammary gland adenomas and intestinal neoplasms (34). Apc^{Min}-mutant mice that lack the *Tcf*1 gene develop a 100% incidence of spontaneous mammary tumors by 2 months of age, a dramatic reduction in latency compared to mice with either genetic defect alone. These mice also had a decreased latency and increased multiplicity of intestinal

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